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PROTEASE INHIBITORS OF FODDER PLANTS AS A FACTOR OF IMMUNE RESPONSE INFLUENCING THE PHYSIOLOGICAL STATE OF THE POTATO LADYBIRD BEETLE *HENOSEPILACHNA VIGINTIOCTOMACULATA* (COLEOPTERA: COCCINELLIDAE)

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Summary. The paper analyzes how protease inhibitors of fodder plants with different genotypes influenced the physiological state of the potato ladybird beetles that fed on them. Feeding on plants with resistant genotypes leads to malnutrition in phytophagous insects because the nutrient absorption is impaired by the inhibitors of digestive enzymes. This results in a slowdown in the developmental timing and fat accumulation and the induction of various developmental disorders during the ontogeny to the extent of the cessation of vital functions.

Key words: 28-spotted potato ladybird beetle, physiology, immune response, fodder plants, protease inhibitors, Russian Far East.

Н. В. Мацишина, М. В. Ермак, П. В. Фисенко, О. А. Собко, А. А. Гисюк. Ингибиторы протеаз кормовых растений как фактор иммунного ответа, влияющий на физиологическое состояние картофельной божьей коровки *Henosepilachna vigintioctomaculata* (Coleoptera: Coccinellidae) // Дальневосточный энтомолог. 2024. N 492. С. 15-24.

Резюме. В статье приводятся данные о влиянии ингибиторов протеаз кормовых растений с разными генотипами на физиологическое состояние картофельной коровки. Питание растениями с устойчивыми генотипами приводит к голоданию насекомых-фитофагов, поскольку под действием ингибиторов пищеварительных ферментов нарушается усвоение питательных веществ насекомыми. Это вызывает замедление сроков развития и нажировочного питания, а также возникновение различных нарушений развития в онтогенезе вплоть до прекращения жизненно важных функций.

INTRODUCTION

Nutrition is the oldest connection between animals and their environment. For this reason, adaptations to food are so advanced and diversified (Ugolev, 1961). Fodder for insects is a complex mixture of macronutrients, minerals, other compounds, and water (Cohen, 2015). Some compounds are essential nutrients; others do not have a direct nutritive function. There are certain evolutionary relationships between insect pests and plants (Burov *et al*., 2012). Phytophagous insects can be divided in several categories based on their feeding specialization (Slepyana, 1975): host, topical, and ontogenetic specificity (Vilkova & Fasulati, 2001). The category of host specificity includes the specificity of arthropod species to intraspecific forms of fodder plants – varieties, hybrids, and lines (Vilkova & Ivashchenko, 2001). This category unites insect pests of plants and entomophagous generalists (Chernyshev, 2001). The feeding specialization of phytophagous insects depends on the physiological and biochemical characteristics of both plant recipients and phytophagous insects (Vilkova *et al.,* 2015). An important evolutionary characteristic of insects is the ability of phytophages to actively search for optimal conditions for feeding and reproduction (Stepanycheva *et al*., 2007). In particular, many insects adapted to feeding and reproduction on certain organs and tissues of plants only at certain stages of plant ontogeny. The search for fodder plants, feeding, digestion, and nutrient absorption require considerable energy (Burov *et al*., 2012). Correspondingly, plant recipients possess a number of specific (morphological, physiological, etc.) characteristics that protect their tissues against phytophagous insects (Razdoburdin, 2012). Feeding on plants with resistant genotypes requires much more time and, therefore, energy for the biochemical reactions inside a pathogenic organism. From the environmental perspective, the specificity of phytophagous insects to fodder plants can be viewed as a way to preserve and maintain the stability of the system "autotroph – heterotroph" (Vilkova, 2000). It should be noted that plant populations – fodder of insects – are characterized by significant genetic diversity under natural conditions and can be considered as populations of diversified genotypes with differences in their immune responses. This complicates a study on the mechanisms of allelochemical interactions in such trophic systems. Meanwhile, agricultural crops, especially the ones that propagate vegetatively, have a limited number of genotypes or even a single genotype within one variety. Thus, a variety of such crop can be viewed as a population of one genotype. This fact makes cultivated plants a convenient model object for studying the mechanisms of interactions in trophic systems because all plants of the same variety/genotype will have similar immune responses. Differences will be observed only between varieties, i.e. populations, each one of which will be represented by a certain genotype.

The goal of present research is to study how protease inhibitors of fodder plants with different genotypes influenced the physiological state of the potato ladybird beetles that fed on them.

MATERIAL AND METHODS

The research was carried out under laboratory conditions in 2019–2023. Standard methods were employed for maintaining and propagating insect cultures to optimize environmental parameters, population density, and food supply (Zlotin, 1989; Wang *et al*., 2018). In total, 35 thousand insects at different developmental stages were used in the long-term experiment. The following thirteen potato varieties of traditional breeding origin were tested: Smak, Yubilar, Kazachok, Sante, Dachnyi, Avgustin, Yantar, Laperla, Lilly, Queen Anne, Red Lady, Labella, and Belmonda.

Identifying the species of the studied insects. One hundred and four individual beetles, which were collected in Primorsky krai and Amurskaya oblast, served as the material for our research. Species-specific molecular markers of the gene COI mtDNA were used for identification (Guo *et al*., 2022).

Studying the hemolymph of the potato ladybird beetle. The hemolymph of the potato ladybird beetle was obtained using modified standard methods (Shoven, 1953; Zlotin, 1989; Prisnyi, 2016) and studied under a MERLIN microscope (Zeiss).

Analyzing the parameters of phagocytosis was performed by standard methods. The following parameters were calculated: the phagocytic index (PI) as the percentage of the hemocytes involved in phagocytosis to the total number of phagocytes; phagocytic number (PN) as the mean number of objects engulfed by one active phagocyte (Grebtsova, 2014).

Studying the morphological structures of hemocytes. Hemolymph smears were stained with acetocarmine and acidic eosin-methylene blue followed by azur-eosin (according to Pappenheim), by the Brown and Brenn method modified by Khmelnistkyi, with basic fuchsin according to Pfeiffer, and with hematoxylin according to Delafield (Menshikova *et al*., 2016; Haszcz, 2016). The analysis of the images was performed using CellProfiler (Carpenter *et al*., 2006).

Studying the demographic parameters of the population of the potato ladybird beetle. The research data were analyzed using a procedure developed for a mortality table with two sexes (Chi, 1985). The analysis of the mortality table was simplified employing TWOSEX-MSChart (Chi, 2017).

Evaluating the influence of the studied potato varieties on the potato ladybird beetle. To study the influence of the potato varieties on the potato ladybird beetle, we used only hatched and active larvae from the laboratory colony with an emergence rate close to 100% (Vilkova *et al*., 2003) and without any disease symptoms. The developmental timing and survival rate of the larvae was calculated at each stage. All experiments were conducted with three repetitions.

Studying the activity of potato protease inhibitors and the proteases of the potato ladybird beetle. Larvae of the first summer generation collected under field conditions were used to study the activity of proteinase inhibitors and the proteases of the potato ladybird beetle (Shpirnaya *et al*., 2006). We determined the activity of proteinase inhibitors and the proteases of the pest spectrophotometrically by recording changes in the rate of substrate hydrolysis (ΔA/min) with trypsin, a-chymotrypsin and papain in the presence of inhibitor-proteins from different tissues of potato plants (Dao & Friedman, 1994).

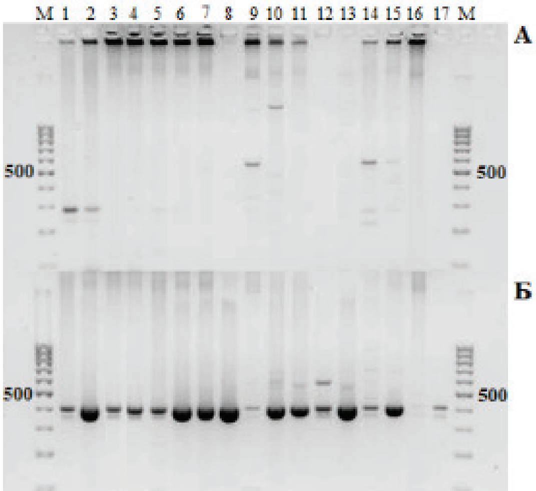


Fig. 1. Analysis of the population of the potato ladybird beetle with the species-specific PCR-markers of the gene COI mtDNA. A – species-specific marker for *H. vigintioctopunctata*, 400 b.p.; Б – species-specific marker for *H. vigintioctomaculata*, 406 b.p.; M – marker of the lengths of fragments 100 b.p. ladder; 1–3 – Primorsky krai: Chuguevsky district; 4–6 – Amurskaya oblast; 7–17 – Primorsky krai: Timiryazevsky.

RESULTS AND DISCUSSION

There are no conclusive data on the species composition of the population of the 28-spotted potato ladybird beetle in literature sources (Kurisaki, 1932; Kawabe, 1947; Ikemoto, 1955; Koyama, 1962; Katakura, 1973.). For this reason, our first step was to ensure that the experiment would be conducted on one species and not on a complex of related species. According to our data, the 28-spotted potato ladybird beetle is represented exclusively by *Henosepilachna vigintioctomaculata* in Primorsky krai. This conclusion was supported by a PCR analysis of a sample from the population of Primorsky krai, Amurskaya oblast, and the south of Sakhalin. The only the species-specific marker of *H. vigintioctomaculata* was amplified (Fig. 1).

Our research showed that the food factor of different origin significantly influenced the morphological development of the phytophage. When potato ladybird beetles fed on alternative food sources their ontogenetic timing and morphological characteristics changed (Matsishina *et al*., 2021). However, the widest range of physiological responses was observed when the phytophagous insect fed on the potato varieties that had various immune mechanisms for protection against the potato ladybird beetle, including the accumulation of specific secondary metabolites, which inhibited the growth and delayed the developmental timing of the potato ladybird beetle. One of the key factors that influenced the feed efficiency and led to malnutrition was the accumulation of the inhibitors of digestive enzymes in the tissues of the phytophagous insect (Matsishina *et al*., 2023). This hypothesis was supported by our experimental data on the activity of the proteases of the potato ladybird beetle (Figs. 2–4). When the potato ladybird beetles fed on potato varieties Smak, Yantar', and Red Lady the activity of invertase and α-amylase was observed to decrease in their bodies. Meanwhile, the activity of these enzymes was by 3-18 times higher in the beetles that fed on the other varieties. This fact in combination with an increase in adrenaline level

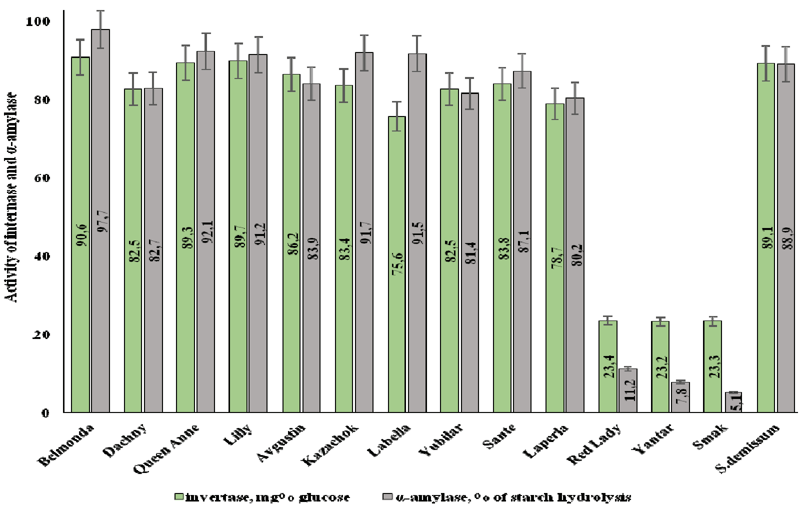


Fig. 2. Activity of internase and α-amylase in the bodies of the larvae of the potato ladybird beetle as a response to their feeding on different potato varieties.

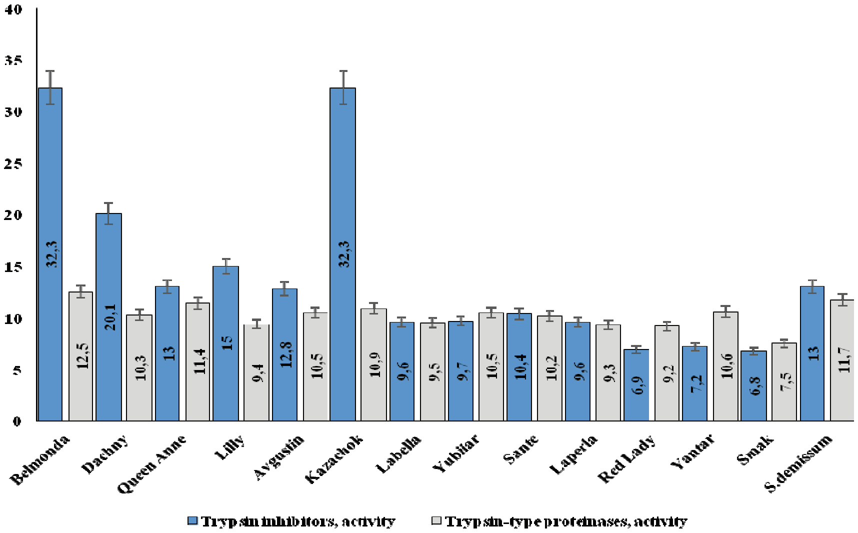


Fig. 3. Sinergetic activity of the protainases of trypsin type (in an insect) and trypsin inhibitors (in a plant) in the course of feeding on different potato varieties.

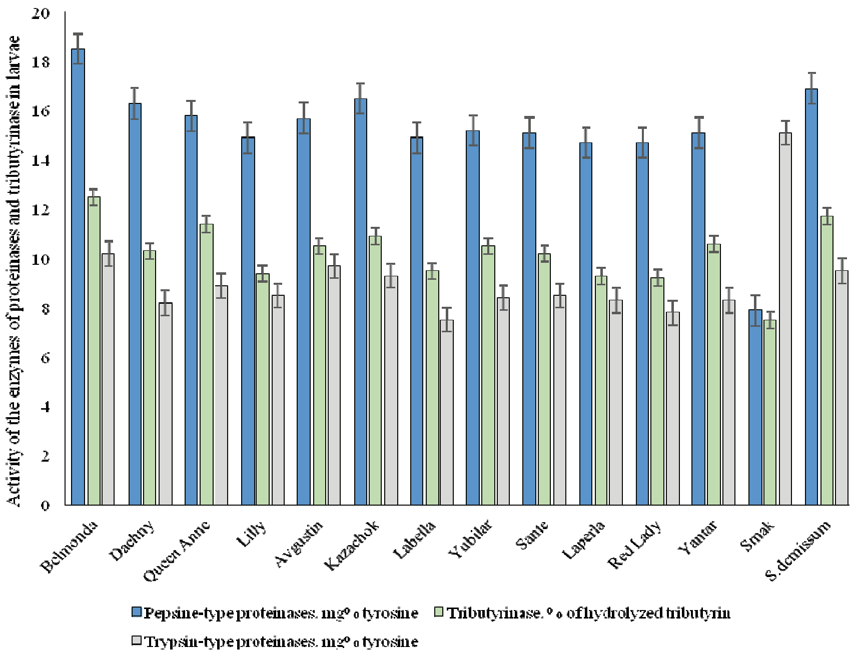
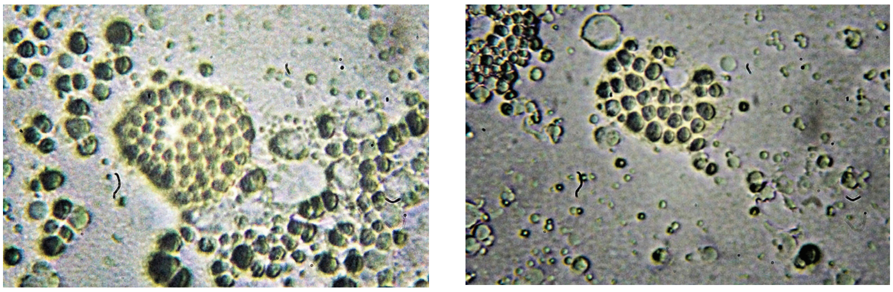


Fig. 4. Activity of the enzymes of proteinases and tributyrinase in larvae of the potato ladybird beetle in response to feeding on different potato varieties.

(Matsishina *et al*., 2023) indicated the slowdown in glycogen synthesis and the activation of glycogenolysis (Mitrofanov, 2007). The highest activity of the lipolytic enzyme (tributyrinase) was detected when the insects fed on potato variety Smak; it exceeded all the other varieties by 1.3–1.8 times. Presumably, when the larvae fed on variety Smak the lipid metabolism remained normal allowing them to effectively use their fat body. At the same time, the utilization efficiency of depot fat decreased in the larvae that fed on all the other varieties except for Smak. This led to the arrested development of their fat body.

The adverse effect of an unfavorable food source (resistant variety) on the potato ladybird beetle is explained by its low nutritional value, which depends on the availability of macronutrients for enzymatic hydrolysis (Shapiro & Vilkova, 1986). Any complications in this process influence the nutrient supply to the hemolymph. All these factors directly affected the ontogeny leading to an increase in the duration of fat accumulation, complicating the transition from one developmental stage to the next, inducing the formation of morphological anomalies in the larvae and imagines, and adding to the total mortality rate (Matsishina *et al*., 2021).



A **B**

Fig. 5.Granulocytes of the potato ladybird beetle in the course of feeding on potato variety Belmonda. A – the norm; B – scattered distribution of granules, cell disruption. Axiolab 5.0, Carl Zeiss, x1000, dark field.

The analysis performed according to Spearman revealed a positive correlation between the parameters "the activity of trypsin inhibitors" and "the activity of the proteinase of trypsin type" ($R=0.2338$, the coefficient of determination is 0.0547, $p \leq 0.01$). Resistant varieties drastically slowed down the feeding of the potato ladybird beetle (Fig. 3). The activity of the digestive enzymes that hydrolyzed proteins, carbohydrates, and fats increased. The hyperfunction of the digestive system reflected a rapid increase in the metabolic cost of nutrient absorption when insects fed on relatively resistant varieties and thus a decrease in the feed efficiency. This indicated a weak oxidation of fatty acids, which complicated the involvement of carbohydrates in the Krebs cycle. As the result, the effect of glucose on lipolysis weakened and the hyperactivation of lipolysis was induced by the synthesis of catecholamines. Extreme stimuli intensify inner processes and increase energy costs demanding a greater amount of lipids and thus leading to the arrested development of the fat body in insects (Rivard *et al*., 2004).

The parameters of the mortality table changed depending on the genotype of fodder plants (Table 1). When the insects fed on beneficial plants (Smak, Yantar, Red Lady, Sante, and Yubilar), most of the parameters were equal to or exceeded the parameter values of the original sample. Feeding on all the other varieties significantly decreased the survivability of the population to the extent of the cessation of vital functions (Belmonda).

Table 1. Influence of potato varieties on the population parameters of the potato ladybird beetle

Population parameters	Original sample	Potato varieties					
		S. demissum	Smak	Yantar	Red Lady	Laperla	Sante
Finite population growth rate, λ (d^{-1})	1.1502± 0.0040	1.0802± 0.0040	1.1605± 0.0040	1.1403± 0.0040	1.1403± 0.0040	1.1302± 0.0040	1.1302± 0.0040
Net reproduction rate, R_0	604.23± 91.71	105.32± 22.31	905.25± 60.48	608.25± 91.71	605.35± 91.71	604.05± 91.71	603.12± 91.71
Average generation time, T(d)	49.05± 0.77	44.02± 0.77	70.02± 0.77	50.01± 0.77	49.03± 0.77	49.03± 0.77	49.05± 0.77
Net consumption, Co (cm^2)	78.12± 5.15	78.12± 5.15	102.012± 6.12	84.12± 5.15	77.15± 5.15	78.12± 5.15	77.10± 5.15

Table 1. Continuation

Population parameters	Potato varieties							
	Yubilar	Labella	Kazachok	Avgustin	Lilly	Queen Anne	Dachnyi	Bel-monda
Finite population growth rate, λ (d^{-1})	1.1205 ± 0.0040	1.1205± 0.0040	1.0802± 0.0040	1.0802± 0.0040	1.0702 ± 0.0040	1.0501 ±0.0040	1.0501± 0.0040	0.0021 ± 0.0040
Net reproduction rate, R_0	604.25 ± 91.71	301.5± 32.01	105.32± 22.31	42.05± 10.02	42.05± 10.02	30.25± 10.02	22.05± 10.02	10.05± 1.05
Average generation time, T(d)	48.05± 0.77	48.02± 0.77	44.05± 0.77	42.02± 0.77	32.03± 0.77	22.04± 0.77	21.03± 0.77	-
Net consumption, Co (cm^2)	76.15± 5.15	75.14± 5.15	75.14± 5.15	75.12± 5.15	42.01± 3.12	32.13 ±2.15	22.01± 1.05	-

Feeding on these plants affected the structural characteristics of hemocytes in the hemolymph of the potato ladybird beetle (Fig. 5). The influence of secondary plant metabolites resulted in a more scattered distribution of granules in granulocytes to the point of cell disruption. Granulocytes lost their ability to grow pseudopodia, the phagocytic index and phagocytic number were zero, i.e. the cells of the hemolymph lost their phagocytic ability.

Thus, food might be a limiting factor for natural populations of the phytophagous insect. On the one hand, the introduction of a new and more nutritious food source such as cultivated potato led to an increase in the population size and habitat range of the potato ladybird beetle in the past (Ermak *et al.*, 2022). On the other hand, feeding on resistant varieties reduces the survivability and fecundity of the pest affecting both the size and diversity of its population.

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